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A Maize Lectin-Like Protein with Antifungal Activity against *Aspergillus flavus*

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ABSTRACT

The filamentous fungus *Aspergillus flavus* causes an ear rot on maize and produces a mycotoxin (aflatoxin) in colonized maize kernels. Aflatoxins are carcinogenic to humans and animals upon ingestion. Aflatoxin contamination results in a large loss of profits and marketable yields for farmers each year. Several research groups have worked to pinpoint sources of resistance to *A. flavus* and the resulting aflatoxin contamination in maize. Some maize genotypes exhibit greater resistance than others. A proteomics approach has recently been used to identify endogenous maize proteins that may be associated with resistance to the fungus. Research has been conducted on cloning, expression, and partial characterization of one such protein, which has a sequence similar to that of cold-regulated proteins. The expressed protein, ZmCORp, exhibited lectin-like hemagglutination activity against fungal conidia and sheep erythrocytes. Quantitative real-time PCR assays revealed that ZmCOR is expressed 50% more in maize kernels from the Mp420 line, a type of maize resistant to *A. flavus,* compared with the expression level of the gene in the susceptible B73 line. ZmCORp exhibited fungistatic activity when conidia from *A. flavus* were exposed to the protein at a final concentration of 18 mM. ZmCORp inhibited the germination of conidia by 80%. A 50% decrease in mycelial growth resulted when germinated conidia were incubated with the protein. The partial characterization of ZmCORp suggests that this protein may play an important role in enhancing kernel resistance to *A. flavus* infection and aflatoxin accumulation.

Aspergillus flavus is a fungal pathogen of several crops, most notably maize but also tree nuts, peanuts, and figs, especially in the southern United States where conditions are particularly favorable for the growth and easy dispersal of this fungus *(8).* Aflatoxins, a group of mycotoxins produced by *A. flavus* and other aspergilli, are potent carcinogens, and their presence in food and feed products reduces the value of these goods and poses serious health risks to humans and domestic animals *(8).* Agricultural losses related to mycotoxins average $630 million to $2.5 billion annually *(26).* The effects of aflatoxin contamination impact farmers directly. An infected crop can mean yield losses, restricted markets, increased transportation costs, increased selling costs, and large amounts of nonmarketable product *(22).* Livestock markets also can be affected if the animals consume contaminated feed, resulting in livestock death, reduced immunity, and suppressed growth and loss in supply efficiency *(22).* Current methods to eliminate postharvest aflatoxin contamination are not economically feasible; thus, research efforts have focused on preharvest prevention of contamination *(14).*

Contamination of a crop with aflatoxins occurs preharvest and during storage. One approach to decrease crop contamination by aflatoxin is through the utilization of host resistance to *A. flavus* colonization *(14, 22).* However, screening for resistant genotypes is difficult because of year-to-year variations in weather and other growth conditions that result in various concentrations of fungal infection and aflatoxin accumulation *(14).* A marker-assisted breeding approach for aflatoxin host resistance can eliminate these problems and reduce the excessive time associated with screening numerous potential maize genotypes by creating one reliable genotype as a lab benchmark *(14).*

Proteomics has been used recently to identify several proteins that occur in higher amounts in maize lines resistant to aflatoxin accumulation *(6).* Several of these identified proteins function as stress-related proteins, such as water stress inducible proteins and heat shock proteins *(6).* One such protein, ZmCORp, which has a sequence similar to that of a cold-regulated protein (COR AJ291295), was found at concentrations twofold or higher in the *A. flavus*–resistant maize line MP420 as compared with the susceptible line B73 *(5, 7).* The relationships among plant stresses such as drought and fungal infection may indicate a link between the stress tolerance of a kernel and its ability to resist *A. flavus* colonization *(6).*

Lectins are proteins that recognize and specifically bind to glycoproteins, glycolipids, or polysaccarides *(9).* This specific binding characteristic allow lectins to function as communicators within and among cells *(9).* Plant lectins are found throughout many different plant species and play a variety of biological roles. Lectins often are synthesized abundantly during seed development and are catabolized into amino acids in growing seeds *(9).* Lectins also may play a role in resistance of plants to fungal infection. When

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the fungal hyphae grow into a plant, they disturb the cellular compartmentalization and cause the release of lectins into the localized area *(9).* Lectins are thought to have evolved with a conserved carbohydrate binding domain, and many possess chitin-binding domains, which is suggestive of their role in plant defense *(9).*

Research into the antifungal and insecticidal properties of lectins is ongoing, and several good reviews regarding the role of plant lectins in the defense against pests are available *(9, 12, 16).* The chitin-binding abilities of lectins aid in the defense of plants against insects and fungi because these proteins are able to penetrate the fungal cell wall and block the active sites of enzymes involved in cell wall production *(2).* The potential of plant lectins to act in the defense of the plant against a fungal pathogen is evident from the activity of these proteins. When fungal hyphae invade plant tissues, the subsequent destruction of compartmentalization in the plant causes the release of these lectins, which deter overall hyphal growth *(9).* For instance, a lectin from *Talisia esculenta* inhibited the growth of several fungi, including *Fusarium oxysporum (13),* and several lectins from *Dolichos biflorus* and *Phaseolus lunatus* inhibited the germination of spores of *Ustilago maydis,* the causal agent of corn smut *(25).* Lectins, especially chitin-binding lectins, are particularly fungicidal in nature and may be triggered by the plant's active defense system; both a wheat germ agglutinin and a barley lectin accumulated in roots infested with the cereal cyst nematode *Heterodera aveneae (16).* The lectin concentration generally dropped 3 days after germination in healthy root tissue, but in nematode-infested tissues lectin concentrations decreased at a much slower rate *(16).*

The present study is part of an ongoing effort to identify and characterize maize proteins potentially involved in the resistance of kernels to *A. flavus* infection. A proteomic approach was used to identify these proteins *(6, 7).* In the present study, one such protein, ZmCORp, accumulated in kernels from maize lines exhibiting increased tolerance to *A. flavus.* ZmCORp was partially characterized. The cDNA encoding ZmCORp from maize was cloned and expressed, and its antifungal properties were investigated.

MATERIALS AND METHODS

Unless otherwise stated, materials were of molecular biology grade and were obtained from Fisher (Pittsburgh, PA).

Identification of genes. A maize cDNA for *ZmCOR* (NCBI accession no. CK986091; http://www.ncbi.nih.gov) was provided by Dr. Hans Bohnert (University of Illinois, Urbana).

Protein expression and purification. The Champion pET Directional TOPO Expression Kit with Lumio Technology from Invitrogen (Carlsbad, CA) was used to express ZmCORp. The primers used were 5′ CACCTACGGCGTGCCCGT 3′ (forward) and 5′ TGTGTTAGTTGTTGGCGACG 3′ (reverse). A CACC overhang was added to the forward primer to ensure directional insertion during cloning. The PCR was conducted with *GoTaq* Flexi DNA polymerase (Promega, Madison, WI) under the following conditions: denaturation at 95°C for 2 min followed by 35 cycles of 1 min at 95°C, 30 s at 55°C, and 1 min at 72°C, with a final extension of 15 min at 72°C.

PCR products amplified from the maize cDNA were cloned into the vector pET160/GW/D-TOPO (Invitrogen) according to the protocol provided. The *Escherichia coli* strain BL21Star was used to express *ZmCOR* with the addition of isopropyl-β-D-thiogalactopyranoside, at a final concentration of 1 mM following the instructions provided by the manufacturer. Protein expression concentrations were assessed at several time intervals to determine the appropriate induction time for specific gene expression.

The resulting samples were separated on a 12.5% (wt/vol) 29:1 bis-acrylamide:acrylamide gel by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and further analyzed through utilization of Lumio technology (Invitrogen). A 2-h induction time was deemed most appropriate for expression of the desired protein, which was isolated in the insoluble fraction. The 6xHis-tagged expressed protein was purified in an Ni-NTA agarose column (Invitrogen). The purified protein was dialyzed against 1 M phosphate buffer (pH 7.0). The final concentration of the protein was determined using a Bradford assay kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions.

Fungal strains and culturing. *A. flavus* strain 3357 was cultured on potato dextrose agar (PDA) plates and incubated at room temperature. Average culture growth time was 2 weeks. To prepare the conidia for assays, plates were scraped lightly with an excess of phosphate-buffered saline (PBS) buffer (pH 7.4), cell counts were recorded, and dilutions of the suspension were made as needed.

Agglutination assays. Agglutination assays were conducted with 1.3×10^5 conidia of *A. flavus* and 5.42 μg of ZmCORp in depression slides in a total volume of 20 μl. Assays with 9.2×10^5 trypsinized sheep erythrocytes (Sigma, St. Louis, MO) were performed to confirm the lectin-like properties of ZmCORp. Lectin from *Phaseolus vulgaris* (Sigma) was used at comparable concentrations as a positive agglutinin control. Agglutination was assayed 30 min after incubation at room temperature.

Germination assays. To ascertain the effect of ZmCORp on fungal germination, 6.8×10^4 conidia of *A. flavus* were incubated with 5.4 μg of ZmCORp in PBS buffer (pH 7.5) (final ZmCORp concentration of 18 mM) for 1 week at room temperature in depression slides placed in a humid chamber. To ascertain the fungicidal effect of ZmCORp, 6.8×10^4 conidia of *A. flavus* were incubated with 5.4 μg of ZmCORp in PBS (pH 7.5) (final ZmCORp concentration of 18 mM) for 1 week at room temperature in 0.5-ml microcentrifugation tubes. The conidia were then washed with fresh PBS twice by centrifugation, and the solution was plated on PDA. The plates were incubated at room temperature for 24 h and the colonies were counted. Assays were conducted in triplicate.

Effect of ZmCORp on elongation of hyphae. To assess the effect of ZmCORp on the elongation of hyphae, an assay was conducted on germinated *A. flavus* conidia. Assay conditions were identical to those described for the germination assays except that conidia were incubated overnight in PBS (pH 7.5) before the ZmCORp was added. Counts were performed with 100 germinated conidia. Assays were conducted in triplicate.

RNA extraction and quantitative reverse transcriptase PCR. Total RNA was extracted from kernels at the dough stage from the maize line MP420, which is resistant to aflatoxin accumulation, and from the maize line B73, which is susceptible to toxin buildup *(6, 7).* Seeds were provided by the U.S. Department of Agriculture Agricultural Research Service (North Central Region Plant Introduction Station, Ames, IA). Plants were grown in

the research plot during the 2007 growing season. Kernels were collected from six ears, each from a different plant. The collected kernels (20 kernels from each ear) were pooled, and RNA was extracted from 5 randomly selected kernels. Total RNA was extracted with acid phenol *(24).* Extracted RNA was then cleaned with the RNeasy kit (Qiagen, Valencia, CA) following the protocol provided by the manufacturer. Extracted RNA was treated with RNase-free DNase RQ1 (Promega) following the provided protocol.

Eight micrograms of total RNA and oligo dT primers (Ambion, Austin, TX) were used to synthesize cDNA with a reverse transcriptase kit (Invitrogen). Real-time PCR was performed in a DNA Engine Opticon 2 system thermocycler (MJ Research, Alameda, CA) with IQ syber green (Bio-Rad, Hercules, CA). The primers used to amplify the ZmCOR fragment were 5′ TACGGCGTGCCCGTG 3′ and 5′ TGTGTTAGTTGTTGGCGACG 3′. An annealing temperature of 55°C was used. The maize actin gene *MAc1* (NCBI accession no. J01238) was used as an internal control to normalize the PCRs. The actin primers used were 5′ GTGACAATGGCACTGGAATG 3′ and 5′ GACCTGACCATCAGGCATCT 3′.

To calculate the difference in ZmCOR expression in cDNA from line Mp420 versus line B73, the procedure described by Livak and Schmittgen *(17)* was followed using the formula $2^{-\Delta\Delta Ct(\Delta Ct\text{B73-}\Delta Ct\text{Mp420})}$. The ΔCt value for each sample was calculated and normalized by subtracting Ct_{actin} from Ct_{ZmCOR}.

Inhibition of agglutination by carbohydrates. Inhibition of the agglutination activity of ZmCORp was tested using the following sugars: D-galactose, D-(+)-mannose, D-fructose, *N*-acetyl-D-glucosamine, D-(+)-glucose, and sucrose. Solutions of the 200 mM sugars were serially diluted with PBS buffer into assays containing 3.28×10^5 *A. flavus* conidia and 5.4 μg of ZmCORp; the final concentration of ZmCORp in the assay was 18 mM. The sugar solution was added to the ZmCORp solution and allowed to incubate at room temperature for 10 min. The conidial suspension was added, and the reaction was placed in a humid chamber and allowed to sit for 30 min. Any sugar that inhibited agglutination by at least 50% was noted.

Temperature and pH stability of ZmCORp. The stability of the recombinant ZmCORp was determined using protocols similar to those used to characterize other plant lectins *(2, 10, 18).* Protein samples (1.05 μg) were incubated at 30, 40, 50, 60, 70, 80, and 90°C for 30 min and then returned to 25°C for agglutination to determine the temperature stability of the protein. ZmCORp samples (1.05 μg) also were incubated in 0.1 M citrate buffer at pH 4.0 and 5.0, in 0.1 M phosphate buffer at pH 6.0 and 7.0, in 0.1 M Tris-HCl buffer at pH 8.0, and in 0.1 M sodium carbonate buffer at pH 9.0, 10.0, 11.0, and 12.0 *(10)* for 1 h, dried in a speed-vac, and subsequently redissolved in PBS buffer (pH 7.8). The agglutination assay was then conducted to determine the pH stability of the ZmCORp. Both assays were conducted in a final volume of 20 μl (final concentration of ZmCORp in the assays was 3.5 mM). Agglutination was scored on a scale of 0 to 4, with 0 representing no agglutination and 4 representing full agglutination.

RESULTS

ZmCORp, a lectin-like protein from maize. A proteomic approach has been used to identify proteins present at higher concentrations in the endosperm of kernels from maize lines resistant to aflatoxin accumulation *(7).* ZmCORp, one of these proteins, was found in at least twofold higher concentrations in the endosperm of kernels from maize lines resistant to *A. flavus* than in the endosperm of kernels from susceptible maize lines. Use of partial peptide sequences (SPPLEWYGVPGGAR and LGGLQEGVNDWK) for ZmCORp and BLAST analysis (http://www.ncbi.nih.gov) led to the identification of a matching cDNA (CK986091). Analysis of the sequence of ZmCOR using Blastx (http://www.ncbi.nih.gov) revealed that the protein sequence was very similar (E values < e-50) to those of various cold response proteins from *Hordeum vulgare* (NCBI accession no. CAC12881), *Oryzae sativa* (NCBI accession no. NP_001055802), and *Triticum aestivum* (NCBI accession no. BAC41494). Amino acid analysis of the predicted protein sequence with the codon usage utility from the Sequence Manipulation Suite Tool (http://www.bioinformatics.org/sms2/codon_usage.html) revealed that the mature protein contains 138 amino acids with high glycine content (Table 1). Codon usage also was biased, with 94.93% of all amino acids ending in G or C (0.72% T, 4.35% A, 46.38% G, and 48.55% C). A screen for relevant protein signatures in ZmCORp using Proscan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = npsa_prosite.html) revealed that the protein harbors the signature sequence LDDeVHLGnk, a common sequence among many identified plant lectins such as those from *P. vulgaris* and *Lablab purpureus.*

TABLE 1. *Amino acid composition of ZmCORp as determined with the codon usage utility from the Sequence Manipulation Suite Tool*

Amino acid	%
Alanine	9.02
Arginine	4.51
Asparagine	3.76
Aspartic acid	6.02
Cysteine	0.00
Glutamic acid	7.52
Glutamine	3.76
Glycine	12.78
Histidine	3.76
Leucine	9.77
Methionine	1.50
Phenylalanine	2.26
Proline	10.53
Serine	3.01
Threonine	2.26
Tryptophan	3.76
Tyrosine	1.50
Valine	10.53

Protein expression and purification. Isopropyl-β-D-thiogalactopyranoside–induced expression of *E. coli* BL21Star transformed with ZmCOR harboring the expression vector and purification of the expressed protein by affinity chromatography using an Ni-NTA column revealed a protein around 20 kDa in size after electrophoresis under denaturing conditions (Fig. 1).

Lectins differ greatly in size, but this 20-kDa protein is similar in size to lectins previously reported, such as the 14-kDa lectin from *Annona coriacea* seeds *(10).* In the absence of ZmCORp antibodies, Lumio staining was used to confirm the identity of the 20-kDa band. The reagent binds

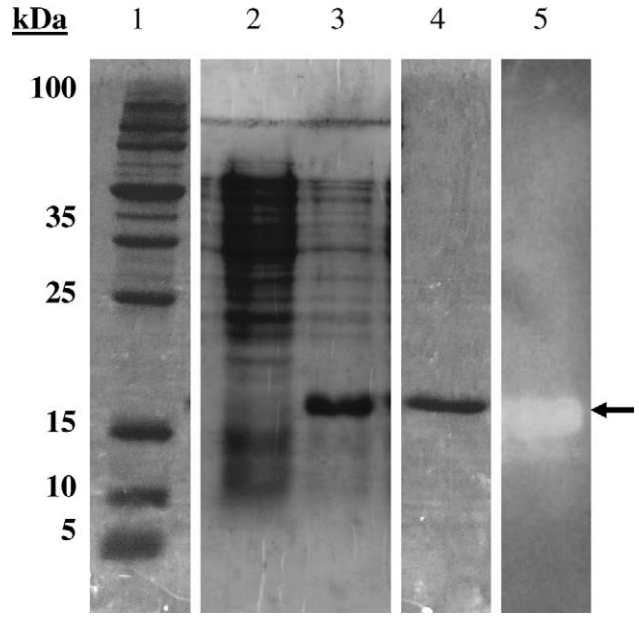

FIGURE 1. *SDS-PAGE analysis of expressed ZmCOR. Lane 1, protein standard (Prosieve); lane 2, crude protein extract (no induction); lane 3, crude protein extract (after induction); lane 4, ZmCORp after passage through an Ni-NTA column; lane 5, confirmation of expression using Lumio staining.*

exclusively to the Lumio tag associated with proteins expressed using the pET160/GW/D-TOPO expression system (Fig. 1).

Agglutination and germination assays. To test the antifungal properties of ZmCORp, germination assays with *A. flavus* conidia were conducted. Germination assays performed with the protein at a final concentration of 18 mM ZmCORp indicated a decrease in the germination of *A. flavus* conidia by approximately 90% as compared with that of conidia in the PBS buffer control (Table 2 and Fig. 2). Germination assays were repeated three times. Because of the presence of a lectin signature sequence in ZmCORp and the visual observations of the protein and its interaction with *A. flavus* conidia, the apparent lectin-like characteristics of ZmCORp were tested. The addition of the expressed ZmCORp at a concentration of 18 mM to a suspension of *A. flavus* conidia resulted in visible agglutination of these conidia after 30 min of incubation at room temperature (Fig. 3); this activity was verified by repeating each assay three times. The lectin agglutinating activity of ZmCORp also was verified by using glutaraldehyde-stabilized sheep erythrocytes as a positive control (data not shown). When macroconidia of *Fusarium verticillioides* and *Fusarium graminearum,* two mycotoxin-producing organisms that cause an ear rot on maize, were treated with ZmCORp under the same previously described experimental conditions, the protein failed to cause any discernible agglutination of the macroconidia. The variation in the effects of lectins on conidia from different species is often attributed to the different sugar-binding specificities of the lectins and to differences in the chemical composition of the coats of the conidia from different species *(11).*

To assess whether the effect of ZmCORp was fungistatic or fungicidal, *A. flavus* conidia were treated with 18 mM ZmCORp as previously described. The conidia were then washed by centrifugation with PBS. The washed conidia were subsequently plated on PDA, and the formed colonies were counted 48 h later. There was no significant difference in the number of colonies formed for the treatment (conidia treated with ZmCORp and then washed) and the control (washed conidia). The experiment was repeated twice. These results indicate that the effect of ZmCORp may be fungistatic under the conditions used. We cannot rule out the possibility of ZmCORp having fungicidal activity under more stringent conditions such as higher concentrations of the protein or longer periods of contact between the fungus and the protein. The antifungal activity of ZmCORp in maize kernels also may be amplified in the presence of other antifungal compounds present in the plant, such as proteinase inhibitors and hydrolytic enzymes *(9, 12).*

TABLE 2. *Effect of ZmCORp on* Aspergillus flavus[a]

Parameter	Control	ZmCORp
Conidial germination (%)[b]	87 ± 4	6 ± 2
Hyphal length (μm)[c]	26 ± 3	14 ± 2

[a] Values are the mean ± standard deviation of three replicates.
[b] 6.8×10^4 conidia were incubated for 1 week with 18 mM ZmCORp.
[c] 6.8×10^4 conidia were germinated overnight and then incubated with 18 mM ZmCORp. Hyphal length was measured 24 h after incubation with ZmCORp. Values are the mean from 100 germinated conidia.

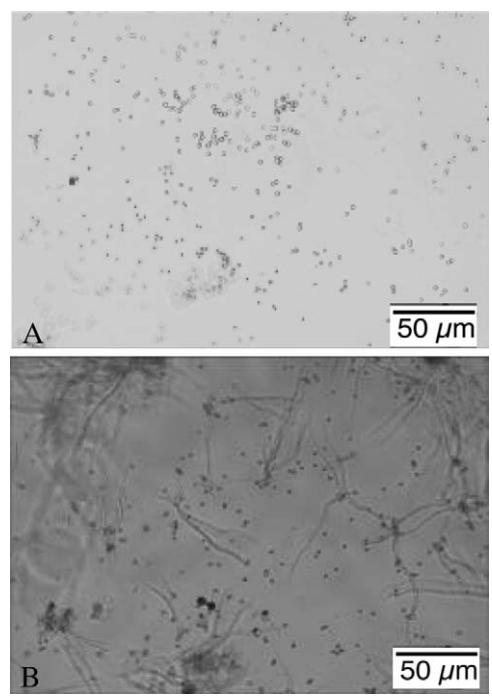

FIGURE 2. *Effect of ZmCORp on the germination of conidia from* A. flavus. *(A) Decreased to no germination was evident in* A. flavus *conidia treated with ZmCORp (18 mM). (B) Germination of conidia in an equivalent volume of PBS buffer.*

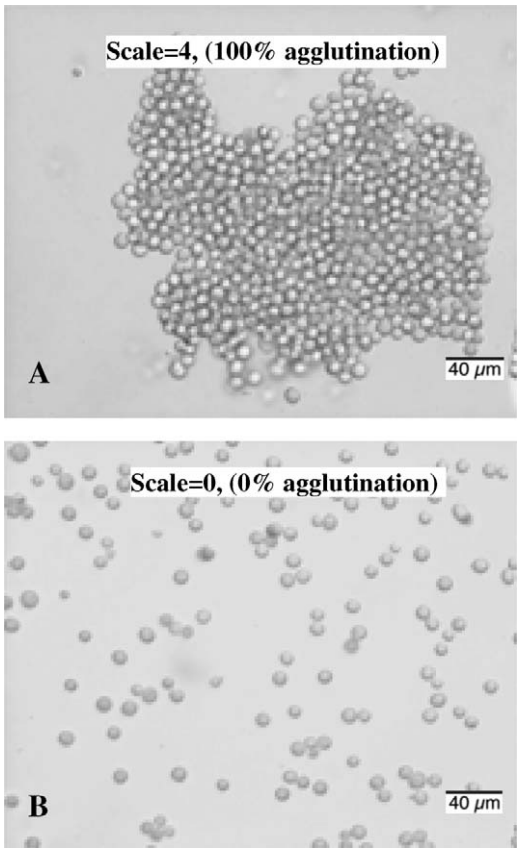

FIGURE 3. *Agglutination assays with conidia of* A. flavus *and ZmCORp treatment. (A) Agglutination was evident in conidia of* A. flavus *treated with 18 mM ZmCORp. (B) No agglutination occurred in an equivalent volume of PBS buffer. Scoring was done on a scale of 0 to 4, with 4 representing full (100%) agglutination (A) and 0 representing no agglutination (B).*

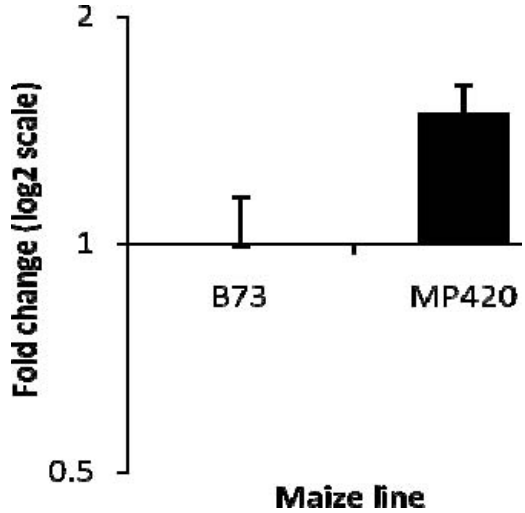

FIGURE 4. *Expression analysis of ZmCOR by quantitative reverse transcriptase PCR. The fold change represents the difference in level of expression of ZmCOR in the resistant maize line MP420 compared with that in the susceptible maize line B73. The* y *axis is shown in a* log_2 *scale. The bars represent the variation among triplicate samples.*

Effect of ZmCORp on germinated conidia. When germinated conidia from *A. flavus* were treated with ZmCORp at a final concentration of 18 mM, the protein affected the growth of the mycelia. The treatment resulted in a 46% reduction in the length of individual hyphae (Table 2). The fact that the antifungal effect of ZmCORp is not restricted to its effect on the germination of conidia is very significant. The presence of ZmCORp in the endosperm of maize may limit the colonization of infected kernels by *A. flavus* by limiting the extent of mycelial growth of the fungus.

Quantitative real-time PCR. Quantitative real-time PCR was conducted on cDNA extracted from maize kernels at the dough stage. ZmCOR expression levels were around 50% higher in kernels from the aflatoxin-resistant maize line Mp420 when compared with the levels in the susceptible line B73 (Fig. 4). This finding is in accord with an earlier proteomic study in which ZmCORp accumulated at least twice as much in the endosperm of kernels from resistant maize lines when compared with kernels from susceptible maize lines *(7).*

Inhibition of agglutination by different carbohydrates. Lectin-binding functions are attributed to the very specific interaction of the protein with its substrate. Therefore, the interaction of a lectin with specific sugars is of interest. Inhibition of agglutination demonstrates lectin activity with a specific sugar and affords the researcher information regarding that reaction and the specificity of the lectin. The addition of various sugars to a solution of ZmCORp and conidia allowed the observation of possible agglutination inhibition due to carbohydrate-binding specificities of ZmCORp (Table 3). The most potent inhibitors were 50 mM *N*-acetyl-D-glucosamine and 50 mM D-(+)-mannose. Another inhibitor was 100 mM D-galactose. All assays were repeated in triplicate, and inhibition was marked by a steep decrease in agglutination (approximately a score of 2 or less) as compared with the control ZmCORp agglutination (score of 4). Sucrose, D-fructose, and D-(+)-glucose did not have an effect on the agglutination caused by ZmCORp.

Temperature and pH stability of ZmCORp. The temperature and pH stability of ZmCORp was tested. The

TABLE 3. *Concentration required for* I_{50} *in the agglutination inhibition assays*[a]

Sugar	Concn (mM)
D-Galactose	100
Sucrose	NI[b]
D-(+)-Glucose	NI
N-Acetyl-D-glucosamine	50
D-Fructose	NI
D-(+)-Mannose	50

[a] Concentration of each sugar required to inhibit by 50% the agglutination of *A. flavus* conidia (3.28×10^5 conidia) treated with 5.42 μg of 18 mM ZmCORp.

[b] NI, no inhibition of agglutination at concentrations up to 100 mM. Assays were repeated in triplicate, and the values presented were consistent in all three replications.

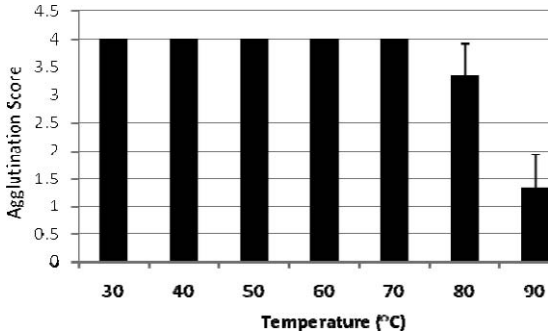

FIGURE 5. *Temperature stability of ZmCORp. Agglutination activity of ZmCORp was assayed on* A. flavus *conidia. Agglutination was ranked on a 0 to 4 scale, with 4 representing full (100%) agglutination and 0 representing no agglutination. Assays were repeated in triplicate; the bars represent the mean of these replications. Error bars correspond to the standard deviation of these replications.*

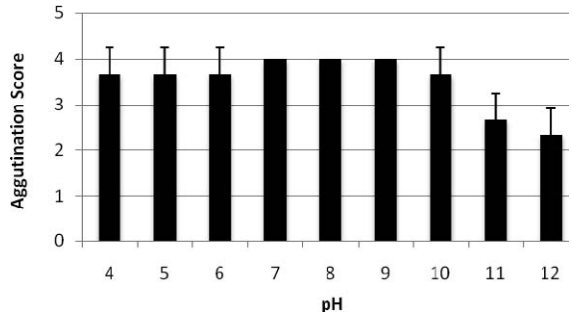

FIGURE 6. *The pH stability of ZmCORp. Agglutination activity of ZmCORp was assayed on* A. flavus *conidia. Agglutination was ranked on a 0 to 4 scale, with 4 representing full (100%) agglutination and 0 representing no agglutination. Assays were repeated in triplicate; the bars represent the mean of these replications. Error bars correspond to the standard deviation of these replications.*

ability of a protein to withstand extreme conditions can yield insight into its composition and its function within the plant. The thermal stability of ZmCORp is detailed in Figure 5. The ability of the protein to agglutinate *A. flavus* conidia after being subjected to a range of increasing temperatures is shown. ZmCORp remained stable and continued to demonstrate strong agglutination activity after incubation at temperatures of 30 to 80°C. Heating at 90°C and above decreased ZmCORp activity substantially. The activity of ZmCORp after incubation at pHs ranging from 4.0 to 11.0 is shown in Figure 6. Agglutination occurred between pH 4.0 and 10.0 but decreased sharply at pH 11.0 and above.

DISCUSSION

Recent research into genetic markers linked to resistance of plants to pathogens has suggested that a correlation may exist between the tolerance of a plant to stresses such as drought or high temperatures and its disease resistance capability *(5).* Stress-induced response signals and mechanisms act as an attempt by the plant cell to respond appropriately to environmental stress, e.g., drought, unfavorable temperatures, insect infestation, or fungal infection.

In comparisons of kernel proteins produced in lines of maize resistant to aflatoxin accumulation versus lines susceptible to this accumulation, researchers have identified certain proteins that may be responsible for the reported resistance. Several of these proteins, including ZmCORp, have been identified as storage and stress-related proteins *(6).* ZmCORp sequences were highly similar to those of cold-response proteins identified in several plant systems. In our experiments, ZmCORp also exhibited lectin-like agglutination activity. Cold-response proteins include a myriad of proteins produced by plants under general stress conditions, including low ambient temperatures *(5).* However, lectins tend to have a higher degree of expression and accumulation during seedling germination. Lectins also are believed to serve as storage proteins with an important nutritional role in developing seeds *(9).* Some plant lectins have been characterized as having cryoprotective properties *(11).* There is ample evidence of the involvement of some lectins *(8, 11)* in enhancing the resistance of plants to pathogens.

ZmCORp exhibits several characteristics that suggest a possible role of this protein in the resistance of certain maize lines to *A. flavus* infection. ZmCORp is relatively high in glycine, valine, and proline (Table 1). The high number of glycine residues in ZmCORp is a common characteristic of many lectins in which this amino acid is crucial for the hydrogen bonding of these lectins to different sugar moieties *(1).* A high glycine content is also characteristic of chitin-binding proteins. Many of these proteins can inhibit the growth of various pathogenic fungi after the disruption of cellular compartmentalization of plant cells by the colonizing fungal mycelia *(3).* The agglutination activity of ZmCORp was demonstrated via assays with conidia and erythrocytes. The clumping action of the protein, at concentrations comparable to those used in the characterization of other lectins *(10, 21),* and the effect on conidia of *A. flavus* and sheep erythrocytes reveals the lectin-like activity of ZmCORp. ZmCORp also largely inhibited fungal germination and the elongation of hyphae. The antifungal properties of the protein may be due in part to its binding specificities. The binding of the protein to the conidial coat potentially alters the composition of this coat in such a way that germination is no longer possible. Swelling and germination modify the cell surface properties of conidia; swelling occurs before germination, causing the inner wall of the cell surface molecules to be exposed *(4).* The exposure of these inner molecules, often composed of chitin and other fungal polysaccharides, makes them prone to binding when specific lectins are present. Affinity of ZmCORp to the chitin present in developing mycelia may lead to the cross-linking of the developing mycelia thus limiting fungal growth *(8).* The apparent specificity of ZmCORp for *A. flavus* conidia and its lack of activity against macroconidia from *F. graminearum* and *F. verticillioides* warrant more studies. This specificity may provide clues regarding the chemical composition of the coats of *A. flavus* conidia. The assays in which the antifungal properties of ZmCORp were investigated were conducted

in the presence of up to 5.4 μg of the protein (a final concentration of 18 mM), which is well within the range of the levels of lectins and other storage proteins that have been isolated from seeds. In an attempt to isolate and characterize salt-soluble lectins in maize, Jankovic et al. *(15)* reported the isolation of two lectins, ZMA-I and ZMA-II, from maize kernels with respective yields of 26 and 16 μg per kernel. The actual amount of ZmCORp needed to inhibit the growth of *A. flavus* in kernels may be less than that used in our assay, given the high compartmentalization of lectins and other storage proteins in maize kernels *(8)*. Our assay results may have underestimated the specific activity of ZmCORp because mannose-specific lectins, such as ZmCORp, tend to aggregate upon isolation (or expression in our case), which tends to reduce their activity *(15)*.

The sugar inhibition assay results indicate that ZmCORp may belong to a group of lectins similar to the maize coleoptyle lectin (CCL) *(21)*. The inhibition of ZmCORp agglutination activity by *N*-acetyl-D-glucosamine, D-(+)-mannose, and D-galactose is very similar to the results obtained from research on CCL, which is galactose specific *(21)*. The interaction of ZmCORp with *N*-acetyl-D-glucosamine is of special importance because chitin, the building block of fungal cell walls, is composed of *N*-acetyl-D-glucosamine. The interaction of ZmCORp with *N*-acetyl-D-glucosamine would thus limit its bioavailability, restraining the growth of an invading fungus such as *A. flavus* by interfering with its cell wall formation.

The physical characteristics of ZmCORp also appear to be similar to those previously reported for other lectins from plants. The gradual loss of agglutination activity at temperatures 80°C and above and around pH 11.0 was likely due to the protein denaturing. This activity profile is very similar to that of a previously documented and characterized insecticidal lectin from *Koelreuteria paniculata* seeds, which also was strongly inhibited by N-acetyl-D-glucosamine *(18)* and the *Zea mays* CCL, was highly thermostable, and lost agglutination activity only after incubation at temperatures of 80°C and above *(21)*. The sudden loss of agglutination activity at temperatures of 90°C and above also is a common occurrence among other plant lectins, such as the lectin from *Saraca indica,* which is stable at temperatures up to 95°C *(23)*. ZmCORp appears to have a pH stability profile similar to that of a lectin from *Sphenostyles stenocarpa*; this lectin remained active from a pH of 2.0 to a pH of 10.0 *(20)*. A lectin from *T. esculenta* maintained agglutinating activity at a pH as low as 3.0 *(19)*. ZmCORp appears to behave in a manner similar to that of other characterized plant lectins.

We identified a new lectin-like protein from maize kernels, ZmCORp, that has antifungal activity against *A. flavus*. Further studies are required to continue characterizing the role of this protein in the resistance of maize to *A. flavus* infection. This research may include expression studies where the expression levels and the regulation of the expression of ZmCORp are studied across several lines of maize. Alternatively, the patterns of ZmCORp accumulation in kernels from different lines of maize also may shed light on the role of the protein in the resistance of maize to *A. flavus* infection.

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